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(57) Abstract

A method for determining antibodies to two or more different pathogens in a liquid test sample, which comprises capturing the antibodies on a solid phase on which are immobilized antibodies to one or more classes of immunoglobulins, especially a mixture of anti-IgG and anti-IgM antibodies, and determining any antibodies captured. The method may be applied to the determination of other non-crossreacting antibodies and may also be applied to the simultaneous but separate determination of antibodies to different pathogens.

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# IMMUNOASSAY

The present invention relates to immunoassays.

Testing of samples of body fluids and of solid samples, for example, of cells or tissue, obtained from a body for the presence of various organisms, in particular bacteria, viruses, parasites and other pathogenic (infectious) organisms, is carried out routinely. Testing is carried out in two main contexts. One is testing of samples of body fluids and of solid samples for the purpose of diagnosing disease, monitoring the course of disease and/or monitoring treatment in an individual. This type of testing is often called "clinical" testing. A clinical laboratory typically carries out tests for a large number of different organisms. The term "pathogen" is used herein to denote a disease-causing organism.

Another major type of testing is the screening of donated blood in order to maintain a supply of blood and blood products free from pathogenic contamination. The regulatory authorities in each country specify the pathogens for which testing is to be carried out. In most countries, screening is currently mandatory for HIV, hepatitis C (non-A non-B hepatitis), hepatitis B and syphilis. In some countries there is a further requirement to test for HTLV. In most countries it is currently mandatory to test for both HIV-1 and HIV-2. Blood to be used for transfusion to patients who are to or who have received a transplant or are otherwise immuno-compromised is generally tested for the presence of CMV (cytomegalovirus). As new pathogens and new sub-types of known pathogens that pose a threat to the blood supply are discovered, the mandatory requirements for testing donated blood are extended to those pathogens.

The most widely used tests for blood screening are immunoassays. For most blood viral pathogens, it is more difficult to obtain the required sensitivity when testing blood for the presence of antigens than it is when testing for the presence of antibodies. Generally the tests for HIV, HTLV, HCV and CMV are antibody tests. There are two tests for hepatitis B: an antigen assay for

hepatitis B surface antigen (HBsAg), and a test for antibodies to hepatitis B core antigen (HBc). At present, HBsAg testing is mandatory in most countries; testing for hepatitis B core antibodies is mandatory in some countries and may be introduced in more. There are both antibody and antigen tests for syphilis.

Blood screening is carried out on a large scale, and it is particularly important that the results are obtained quickly, since blood has a relatively short shelf-life. In order to save both time and money, assays capable of detecting more than one pathogen in a single test sample have been proposed.

Antibody assays for determining two sub-types of the same pathogen, in particular, for HIV-1 and HIV-2 (HIV-1+2), and also for HTLV-I and HTLV-II (HTLV-I+II) are on the market. It is to be noted, however, that there is often considerable cross-reactivity between sub-types of the same species, for example, the degree of immunological cross-reactivity between HIV-1 and HIV-2 is such that the HIV-1 tests initially on the market did, in fact, detect a very large proportion of HIV-2-containing blood. Accordingly, as is also explained in EP-A-0 484 787, there is a very considerable technical difference between providing an antibody assay for two sub-types of the same virus and an antibody assay for two completely different pathogens having essentially no immunological cross-reactivity. The term "combination assay" is used herein to denote an assay for the detection or determination, in a single test on a sample, of two or more pathogenic organisms that have substantially no immunological cross-reactivity. The term "different pathogens" is used herein to denote pathogens that have substantially no immunological cross-reactivity.

EP-A-0 484 787 proposes combination antibody assays for viral pathogens that may occur in donated blood (blood viral pathogens), in particular, a combination assay for HIV and HCV (hepatitis C virus). The proposed heterogeneous phase assay involves the use of multiple peptides coated on a solid surface

to capture the relevant antibodies. The resulting antigen-antibody complexes may then be detected.

A major disadvantage of such a combination assay, and also of the HIV-1+2 and HTLV-I+II combination blood assays currently on the market, is the requirement for co-coating a plurality of antigens on a solid surface. Not only must the antigens (peptides and/or proteins) be coated evenly, they must also be coated in such a manner that the epitopes are available for antibody binding. Furthermore, there must be no interaction between the various antigens. The quality control testing that must be carried out to ensure that the antigens have been coated in a satisfactory manner adds to the expense, both in the cost of the testing itself and in the cost of the rejects. The more antigens there are to be coated, the worse the problems and the greater the expense. Each additional pathogen to be detected requires the presence of at least one additional immobilized antigen. For some pathogens it is necessary to enable the detection of multiple antibodies. In the case of HCV, for example, current commercially available assays comprise four different antigens. For a combination HIV-1+2/HCV assay at least six antigens are required.

In addition to the practical manufacturing problems of co-coating, it can be difficult to obtain the required specificity. Very high purity antigens having strong antigenicity are required, see EP-A-0 484 787.

Antibody/antigen combination assays have been proposed for HIV and hepatitis B surface antigen. EP-A-0 286 264, for example, proposes coating a solid phase with a peptide capable of binding to an HIV antibody and coating a solid phase with an antibody specific to HBsAg. The two solid phases may be different, or both the peptide and the antibody may be coated on the same solid phase. WO91/10747 proposes improvements to the type of assay described in EP-A-0 286 264, the improvements being in the nature of the peptides used for coating and for detection of the HIV

antibody, and in the nature of the detection system.

It is noted, however, that in the only commercially available combination assay for HIV and HBsAg based on the principle disclosed in EP-A-0 286 264, the various problems associated with co-coating are circumvented by coating the HIV peptides on the inner surface of small tubes and the anti-HBsAg antibody on beads to be used in conjunction with the tubes. Furthermore, that assay is not a true combination assay in the present sense because, after bringing the sample into contact with the beads in the tubes, the beads are removed and the detection steps are carried out separately on the beads and the tubes. Accordingly, any potential problems regarding interactions between the labelled antibody and the labelled antigen used for detection, and any potential problems between the detection systems (labels) during the detection of any immune complexes formed are avoided.

According to the present invention, the problems of and resulting from co-coating multiple antigens on a solid surface for a combination antibody assay are overcome in a simple and elegant manner by using an immunoglobulin capture format.

The immunoglobulin capture format has been known as such for many years; it was described in 1979 by Duermeyer W. et al in Journal of Medical Virology 4:25-32 for the detection of specific IgM antibodies in an ELISA for hepatitis A. The principle of the described assay for a sample obtained from a human is that a solid phase, for example, the wells of a microtitre plate, are coated with anti-human IgM, sample is added to the coated wells and incubated, the wells are washed and then antigen is added to the wells and incubated, and finally any bound antigen is detected using enzyme-labelled antibody. The authors of that paper proposed the use of the assay for any individual IgM antibodies, for example, against rubella, herpes simplex, CMV, EBV, Toxoplasma gondii, hepatitis B core antigen or other antigens.

Assays using the immunoglobulin capture technique for the detection of single pathogens in blood, for example, a rubella assay and a hepatitis A assay have been available for several years.

WO88/07680 discloses the application of the immunoglobulin capture technique to the determination of antibodies in body fluids other than blood, in particular saliva and urine. A commercially available HIV-1+2 assay for saliva and urine uses the IgG capture technique described.

It is believed that there is a general prejudice in the art against the immunoglobulin capture technique, as demonstrated by the fact that although the technique has been available since 1979 only a very limited number of assays using the technique are available commercially compared to the large number of commercially available assays using antigen to capture antibodies. In particular, it is believed that there is a general perception that, in practice, the technique would not work effectively, either with regard to specificity or with regard to sensitivity.

With the immunoglobulin capture technique, a considerable level of undesired interactions would be expected, leading to lack of specificity. WO89/12231, for example, which describes an antibody capture assay for multiple antibody detection, expresses concern regarding lack of specificity caused by non-immunological protein-protein interactions between antigens and the Fc region of immunoglobulins, and proposes an immunoglobulin capture assay in which the antibodies coated on the solid surface are anti-Fc antibodies. Immunoglobulins are therefore captured specifically at the Fc region, which is thereby made unavailable for non-specific protein-protein interactions.

Sensitivity is also regarded as a potential problem with immunoglobulin capture assays, for example, the immunoglobulins are not captured selectively, so there is no enrichment of the specific antibody species under investigation. If that specific

antibody species is present in very small amounts, both in absolute terms and relative to the other species of antibody present in the serum sample, it would be expected that the detection of the antibody under investigation would be difficult and unreliable and, in particular, that the assay would lack sensitivity.

Contrary to expectation and surprisingly, the present inventors have found that, using the immunoglobulin capture technique, antibodies to two or more different pathogens in samples under investigation can be detected simultaneously with excellent sensitivity and specificity.

The present invention provides the use of the immunoglobulin capture technique in the determination of antibodies to two or more different pathogens in a single test sample.

Accordingly, the present invention provides a method for determining specific antibodies to two or more different pathogens in a liquid test sample, which comprises

(i) contacting the sample with a solid phase on which are immobilized antibodies to one or more classes of immunoglobulin, whereby immunoglobulins of the respective class or classes present in the sample are captured on the solid phase,

(ii) simultaneously or sequentially contacting the solid phase, on which immunoglobulins from the sample have been captured, with two or more different antigens, each antigen being capable of binding selectively to an antibody specific for one of the pathogens under investigation, each antigen being provided with means capable of providing, directly or indirectly, a detectable signal, and

(iii) determining any resulting immunoglobulin-antigen complex formed on the solid phase.

The immobilized antibodies may be directed against IgG, IgM or IgA, or a mixture of antibodies against different classes of immunoglobulin may be used. A mixture of anti-IgG and anti-IgM antibodies is particularly preferred.

The assay of the present invention, a "combination assay" has unlimited versatility because of the use of a universal solid phase for the capture of antibodies. The user can chose which antibodies and hence which pathogens are to be detected in a sample under investigation simply by using the appropriate antigen reagents for detection. This is in complete contrast to the conventional assays that use antigens to capture antibodies, where a different antigen-coated solid phase is required for each combination of antibodies under investigation.

The versatility of the use of a universal solid phase is a major advantage both to the manufacturer and to the user of assays for multiple pathogens, particularly blood screening assays and clinical assays. As pointed out above, a universal antibody-coated solid phase, for example, coated beads or coated wells of microtitre plates, can be used for antibody testing for any and all combinations of pathogens because the selectivity of any particular antibody assay is determined by the combination of antigen reagents used for detection.

This versatility is an advantage for the manufacturer of assays because a universal antibody-coated solid phase may be used for all combination antibody assays, whether they are for blood screening or for clinical testing. At present, the mandatory requirements for antibody testing of donated blood vary from country to country. Different antigen-coated solid phases are therefore required for different territories. The provision of a universal antibody-coated solid phase for all territories reduces manufacturing costs significantly. For clinical testing, a different antigen-coated solid phase is required for each and every different combination antibody assay. Again, provision of a universal antibody-coated solid phase reduces manufacturing

costs significantly.

A further advantage to the manufacturer is the reduction in the number of components to be coated on the solid phase and hence a reduction in manufacturing costs. Assays using antigens to capture antibodies require multiple antigens for combination antibody assays for as few as two pathogens. For example, at least six different antigens are required for a combination HCV/HIV-1+2 assay. The more pathogens to be detected, the more antigens must be coated on the solid phase. According to the present invention, however, it is not necessary to immobilize additional antibodies on the solid phase in order to detect additional pathogens; the presence of anti-IgG alone enables the detection of multiple pathogens. Furthermore, the antibodies used for coating are generally cheaper than the high purity antigens required for successful coating, especially for multiple antigen coating.

The versatility of the combination assay of the present invention is also an advantage for the user because, for example, testing for any particular combination of pathogens may be done simply by using the appropriate combination of antigen reagents. This is particularly useful in the clinical laboratory since only one, universal antibody-coated solid phase need be purchased and stored to enable testing for any desired combination of pathogens.

For screening purposes, it is not necessary to know the nature of a contaminating organism. In the case of blood screening, if a sample of blood is positive for antibodies to any of the proscribed pathogens, that blood is unsuitable for use for transfusion or for the production of blood products, for example, plasma or Factor VIII, and should be discarded.

The ability to test a sample of donated blood in one assay for all the blood viral antibodies laid down by any particular national regulation is a major advance, providing blood banks

with very substantial savings in both time and money. The time saving is important not just in saving the time of technicians and hence being a further cost saving, it is also important in view of the relatively short shelf-life of whole blood.

The immunoglobulin capture assay of the present invention has excellent specificity and sensitivity. We have obtained results for samples containing a mixture of antibodies of interest that are substantially the sum of the results obtained for the corresponding serum or plasma samples each containing only one of the antibody species. It could not have been predicted that, in the combination assay of the present invention, each of a plurality of antibodies, directed to different organisms, reacts as if it were the only antibody species present. The observation of an additive effect with regard to the number of antibody species detected is an unexpected but major practical advantage. It is also unexpected that the assay of the present invention, which can be conducted using a conventional assay format, for example, a microtitre plate, is so sensitive.

An assay of the present invention may be an IgG assay, an IgM assay or, for special purposes, an IgA assay. Alternatively, a mixture of antibodies to immunoglobulins of different classes may be used for coating the solid phase. It is particularly preferred to use a mixture of anti-IgG and anti-IgM antibodies.

A further important advantage of the assay of the present invention compared with assays using coated antigens for antibody capture is that, by using immobilized anti-IgM, early infection may be detected reliably. This is important both for blood screening and for clinical investigations.

IgM is the first class of immunoglobulin to be produced in response to infection. Specific IgM antibody levels rise in the first few weeks after infection, then specific IgG is produced a little later. The IgG antibody response is persistent and may last for many years, even for life. In theory, IgM can be

detected in conventional assays where an antibody of interest is captured onto an antigen-coated surface and the presence of captured antibody is detected using, for example, labelled anti-human-IgM antibodies. In practice, however, such IgM assays generally lack specificity because IgM is "sticky", that is to say, IgM tends to bind non-specifically to the capturing antigen. The labelled anti-IgM antibody used for detection of antigen-IgM complex cannot distinguish between specific antigen-IgM complexes and non-specifically bound IgM. For this reason, conventional assays using immobilized antigens generally use anti-IgG antibodies for detection i.e. the assays are for IgG only.

In the assay of the present invention, anti-IgM antibodies may be coated on the solid phase. Those antibodies then capture IgM immunoglobulins specifically from the sample, and the captured IgM is detected specifically by means of the labelled antigen. We have found, surprisingly, that the system is not "sticky" and that specificity is not a problem as it is with the conventional antigen-capture assays for the detection of IgM antibodies. As indicated above, it is particularly preferable to use a mixture of anti-IgG antibodies and anti-IgM antibodies for coating the solid phase. The presence of anti-IgM antibodies on the solid phase will ensure that any early antibodies present in a sample are detected, and the presence of anti-IgG antibodies will ensure that persistent antibodies are detected.

The antibodies immobilized on the solid phase may be polyclonal antibodies, for example, polyclonal anti-human antibodies, which will contain antibodies to all immunoglobulin classes. Alternatively polyclonal antibodies directed against a particular class of immunoglubulin may be used, for example, polyclonal anti-IgG and polyclonal anti-IgM antibodies. Polyclonal antibodies may be purified, for example, by affinity chromatography. If desired, monoclonal antibodies may be used. Anti-immunoglobulins may be specific for the immunoglobulin gamma, mu or alpha chains of IgG, IgM and IgA, respectively.

Polyclonal and monoclonal antibodies used for coating the solid phase may be prepared by methods known per se. Various anti-immunoglobulins are available commercially, for example, rabbit, sheep and goat polyclonal anti-IgG and anti-IgM immunoglobulins, and mouse monoclonal anti-IgG and anti-IgM immunoglobulins. If a mixture of different antibodies is used for coating, the proportions of the different antibodies in the mixture may be varied as desired. The present invention is not limited to the investigation of samples from humans and also finds veterinary applications. Accordingly, the antibodies used for coating should be directed against immunoglobulins of the species from which the sample under investigation is obtained. In the case of samples from humans, for example, a coating antibody should be an anti-human antibody.

As mentioned above, a particularly advantageous feature of the assay of the present invention is that a universal antibody-coated solid phase means is used regardless of the number or nature of pathogens under investigation. The antibodies used as coating antibodies are directed against one or more classes of immunoglobulins, especially against IgG and IgM, and capture a representative proportion of immunoglobulins of the respective classes from the sample. By choosing any particular combination of specific antigens, the user of the assay can test for any combination of pathogens. The antigen reagents can be provided ready mixed, or the user can use separate antigen reagents in any desired combination. If used individually, the antigen reagents may be contacted simultaneously or sequentially in any order with the solid surface carrying the captured immunoglobulins. It is generally more convenient to add the desired mixture of reagents in one step.

An antigen to be used in an assay of the invention may be any antigenic entity that interacts selectively with the antibody under investigation. For example, an antigen may be a peptide or polypeptide, and may be a synthetic or recombinant antigen, or a purified antigen from cell culture, for example, from a viral

lysate. It may be particularly useful in certain cases, for example, HCV, to use a recombinant fusion polypeptide that comprises more than one antigenic region of a particular organism.

An antigen may itself be labelled with means capable directly or indirectly of providing a detectable signal to enable any immunoglobulin-antigen complex to be detected. The labelled antigen is called an "antigen conjugate".

The detectable signal may be optical, radio-active or physico-chemical, and may be provided directly by labelling the antigen, for example, with a dye, coloured particle, radiolabel, electroactive species, magnetically resonant species or fluorophore; or indirectly by labelling the antigen with an enzyme itself capable of giving rise to a measurable change of any sort. Alternatively, the signal may result from agglutination, a diffraction effect or a birefringement effect involving the antigen conjugate.

In a further embodiment, the means capable indirectly of providing a detectable signal comprises an antibody that binds to the antigen. That antibody (Ab1) may be provided with direct or indirect label means as described above for the antigen (single antibody detection system), or it may be labelled by means of yet another antibody (Ab2), which binds to Ab1 and is itself labelled directly or indirectly as described above for the antigen (dual antibody detection system). A single or dual antibody system for detection of captured antibody-antigen complex is particularly useful if it is difficult to produce a suitable antigen conjugate. Some antigen conjugates, for example, tend to be insufficiently stable for commercial use, and some antigens lose antigenicity on conjugation.

The single antibody detection system has the advantage of involving fewer washing and incubation steps, but the disadvantage that it is necessary to produce an antibody conjugate (labelled antibody Ab1) for each antigen to be

detected. The dual antibody detection system has the advantage that, with appropriate choice of Ab1 and Ab2, it is possible to use one antibody conjugate (labelled antibody Ab2) for the detection of all captured antibodies as follows: when each antibody (Ab1) is raised in the same animal species, the second antibody (Ab2) can be an anti-species antibody. For example, if each antibody Ab1 is a sheep antibody, then a labelled anti-sheep antibody can be used as Ab2. The advantage to the user of requiring only one antibody conjugate for detection of all analytes generally outweighs the disadvantage of the need for an extra incubation and extra washing steps, particularly when an automated system is used. The advantage of requiring only one antibody conjugate is another significant advantage to the manufacturer.

A labelling system that is particularly preferred for an antigen or antibody conjugate is an enzyme labelling system, particularly one in which the antigen or antibody is conjugated to an enzyme that catalyses a detectable colour change in the presence of a suitable substrate. This format, the enzyme-linked immunoassay or ELISA, is widely used commercially, and automated instruments are available for carrying out such assays using, for example, microtitre plates, the proprietary "bead" or "IMX" (Trade Mark) systems, or using hollow rods or pipette tips, and computer software is available to process the results obtained.

The enzyme systems used are well known per se, see for example, "ELISA and Other Solid Phase Immunoassays, Theoretical and Practical Aspects" Eds. Kemeny D.M. & Challacombe S.J.,

Examples of typical enzyme systems are those using alkaline phosphatase, ß-galactosidase, urease or peroxidase, for example, horse-radish peroxidase.

The solid phase on which the antibodies are captured is, for example, beads or the wells or cups of microtitre plates, or may be in other forms, for example, as solid or hollow rods or

pipettes, or particles, for example, from 0.1μm to 5mm in diameter. (Such particles are often called "latex" particles, regardless of the material of which they are made.)

A solid phase may be of a plastics or polymeric material, for example, of nitrocellulose, polyvinyl chloride, polystyrene, polyamide, polyvinylidine fluoride or other synthetic polymers. Particles may additionally be of natural polymers, for example, latex or protein. Microtitre plates and beads are used extensively for both blood screening and clinical testing and are widely available commercially.

Other solid phases that may be used include membranes, sheets and strips, for example, of a porous, fibrous or bibulous material, for example, of nylon, polyvinyl chloride or another synthetic polymer, of a natural polymer, for example, cellulose, of a derivatized natural polymer, for example, cellulose acetate or nitrocellulose, or of glass fibres. Paper, for example, diazotized paper may be used. Films and coatings, for example, of fibrous or bibulous material, for example, as described above, may be used as the solid phase.

It is to be understood that the above examples of solid phases are given by way of illustration only, and the invention is not limited to the use of such solid phases. The invention may be practised on any solid phase suitable for use in immunoassays.

A solid phase, for example, a membrane, sheet, strip, film or coating, may be incorporated in a device for the determination of multiple or, more generally, single samples.

The term "assay device" is used herein to denote means for carrying out an immunoassay comprising a solid phase, generally a laminar solid phase, for example, a membrane, sheet, strip, coating, film or other laminar means, on which are immobilized antibodies to one or more classes of immunoglobulin. The immobilized antibodies are preferably present in a defined zone,

called herein the "antigen capture zone".

An assay device may incorporate the solid phase within a rigid support or a housing, which may also comprise some or all of the reagents required for carrying out an assay. Sample is generally applied to an assay device at a predetermined sample application zone, for example, by pouring or dripping the sample on the zone, or by dipping the relevant part of the device into the sample. If the sample application zone is at a different site from the antibody capture zone, the arrangement of device is generally such that antibodies in the sample migrate to the antibody capture zone. The required reagents are then applied in the appropriate order at designated application zones, which may or may not be the same as the sample application zone. Again, if the or any reagent application zone is at a different site from the antibody capture zone, the arrangement of a device is generally such that the reagent(s) migrate to the antibody capture zone, where any antigen-antibody complex formed is detected. All or some of the reagents required for an immunoassay may be incorporated within a device, in liquid or dry form. If so, a device is generally arranged such that interactions between different parts of the device, which interactions may occur automatically during the operation of the device or may be brought about by the user of the device, bring the various reagents into contact with one another in the correct sequence for the immunoassay to be carried out.

A wide variety of assay devices are described in the literature of immunoassays. Examples of membrane devices are described in U.S. Patents Nos. 4,623,461 and 4,693,984. Depending on their design and their speed of action, some assay devices are called "dipsticks" and some are called "rapid assay" devices. A "rapid assay" device generally provides a result within ten minutes of the application of sample. (A typical microtitre plate or bead assay requires incubation steps, and generally takes at least an hour to provide a result.) Accordingly, although assay devices are generally more expensive than microtitre or bead format

assays, they have particular uses in clinical testing, for example, when a result is required rapidly, for example, in the case of emergency surgery.

Assay devices have the particular advantage that they can be used without the need for sophisticated laboratory facilities or even without the need for any laboratory facilities. They may therefore be used for "on the spot" testing, for example, in an emergency room, in a doctor's surgery, in a pharmacy or, in certain cases, for home testing. They are particularly useful in territories where laboratory facilities are few and far between.

As indicated above, the combination assay of the invention is particularly useful for screening donated blood. The assay may therefore be used to test for antibodies to at least two viruses selected from HIV, for example, HIV-1, HIV-2 and HIV subtypes, for example, HIV-1 subtype O; HCV; hepatitis B (antibodies to core antigen); HTLV, for example, HTLV-I and HTLV-II; CMV; EBV (Epstein Barr virus); and optionally also for syphilis. Particularly preferred are combinations of two or more selected from HIV, for example, HIV-1+2; HCV; HTLV, for example, HTLV-I+II; and hepatitis B core (HBc); for example: HIV and HCV; HIV and HTLV; HTLV and HCV; HIV, HCV and HTLV; HIV, HCV and HBc; HIV, HTLV and HBc; HTLV, HCV and HBc; HIV, HCV, HTLV and HBc. Syphilis may be included in any of the above combinations. The combination of choice will be influenced by specific national regulations. In some cases, donated blood may also be tested for further pathogens, for example, for rubella.

Furthermore, the requirements for blood screening are reviewed as new pathogenic agents (new organisms and new subtypes of known organisms) are discovered, and the mandatory assays are extended to cover those pathogens. For example, it is currently required in most countries to test for both HIV-1 and HIV-2. The recent discovery of HIV-1 subtype O has led to the requirement that assays must also detect that subtype. Accordingly, the combinations of pathogens to be detected will almost certainly

enlarge with time. The present invention includes assays for such combinations of pathogens.

If the tube/bead two component system is used, further versatility can be introduced, for example, after contacting the tube and bead with the sample, the bead may be removed and contacted with antigen(s) to one or more different species of pathogen and the tube contacted with a different antigen or combination of antigens.

A further variant of the tube/bead two component assay format is to immobilize one or more classes of anti-immunoglobulin on one of the components and to immobilize anti-hepatitis B surface antigen (anti-HBsAg) on the other component, for example, anti-IgG and optionally also anti-IgM may be coated on the inner surface of the small tubes and anti-HBsAg on the beads, or vice versa. The beads and the tubes may then be used together in a combined HBsAg/multiple antibody assay, especially an assay for HBsAg, HIV, HCV and optionally one or more further analytes selected from hepatitis B (core), HTLV, and syphilis.

Peptides and polypeptides that interact specifically with HIV-1 and HIV-2 are well known and are described, for example, in EP-A-0 347 148. We have found that it is particularly useful with synthetic HIV peptides to block the sulphydryl groups of the cysteine groups with blocking agents, see for example, EP-A-0 307 149, resulting in a labelled peptide, in particular enzyme-labelled peptide, having greater immunological activity in the assay than a corresponding non-blocked peptide.

The antigen to be used for detection of hepatitis B core antibodies is a core antigen. DNA and predicted protein sequences for various hepatitis B serotypes have been published, for example, by Ono et al (1983) Nuc. Acids Res 11, 1747, and suitable peptide and polypeptide core antigens, both synthetic and recombinant, may be derived from published sequences.

Hepatitis C does not, at present, appear to have one immunodominant epitope, and it is preferable to test for antibodies to more than one region. For example, it may be advantageous to use as antigen a fusion protein that comprises more than one epitope, for example, an amino acid sequence from at least one structural region and from at least one non-structural region, for example, as described in GB-A-2,239,245. Antigens from the core and envelope regions are preferred structural antigens. Non-structural antigens may be selected, for example, from the NS3, NS4 and NS5 regions.

An HTLV antigen may be, for example, a p21e or gp46 recombinant protein or a peptide derived from p21e or gp46 (see, for example, US Patent No. 4,743,678). Purified p21e is available from Cambridge Biotech Corporation, 1600 East Gude Drive, Rockville, Md 20850-5300, U.S.A and gp41 from Repligen Corporation, 1 Kendal Square, Building 700, Cambridge, Ma 02139, U.S.A.

For detection of CMV antibodies, there may be used purified cultured CMV core proteins, for example, p66, or recombinant pp150, which is dominant in Western blots. For detection of EBV antibodies, there may be used capsid or early antigen. Purified T. pallidum antigen may be used for the detection of syphilis antibodies.

A combination assay according to the present invention may be used in clinical diagnosis as a preliminary, screening test for several pathogens. If the test is positive, then a separate test for each pathogen may be carried out. If the result is negative, no further action is required. Overall, time and money is saved. An example of such a combination is that used for the so-called "TORCH" screening of pregnant women in many countries. The combination of pathogens under investigation varies from country to country, in analogy to blood screening requirements, but is generally selected from rubella, toxoplasmosis, CMV and herpes. It is particularly advantageous to use a mixture of immobilized anti-IgG and anti-IgM antibodies to ensure that recent infection

is detected.

A combination assay of the present invention presented in an assay device format, especially "rapid assay" device format, is particularly useful when a screening result is required urgently, for example, in the case of emergency surgery. For example, a rapid assay for two or more different blood viral pathogens, for example, as described above, for example, for HIV-1+2 and HBc will inform a surgeon if special precautions are required.

An assay device for combinations of pathogens of interest is of use, for example, for "on the spot" testing in doctors' surgeries to assist with diagnosis and can, overall, save time and money in both clinical laboratory testing and in the avoidance of repeat visits to the surgery. Furthermore, assay devices are useful for testing in situations where laboratory facilities are not readily available, for example, in rural areas and in developing countries. Examples of combinations of pathogens are CMV and HIV; and TB and HIV.

The versatility of the use of a universal antibody-coated solid phase for the detection of a plurality of antigens can be exploited in a further manner in clinical testing. In clinical testing, the object is generally to identify the pathogen(s) in a sample. At present, if a number of samples have to be tested for the presence of various pathogens using the conventional micro-titre plate or bead format, it is customary to prepare aliquots of the various samples and to run a series of assays, each for a different pathogen. This is because the conventional assays use antigen-coated means to capture pathogen-specific antibodies. Accordingly, it is necessary to use a different antigen-coated means, generally either beads or a microtitre plate, for each pathogen. This increases the time taken to obtain results on a particular sample, and also creates a risk that aliquots of samples will be mislaid or confused while all the various tests are being carried out.

Furthermore, the antigen-coated means is generally presented with other reagents, for example, positive and negative controls, wash solutions and diluents, in the form of a kit. The user therefore has to have a large number of different kits.

Using the assay of the present invention, a universal antibody-coated means, for example, microtitre plate, can be used for the simultaneous but separate determination of each of a plurality of pathogens simply by the use of the appropriate antigen reagents. A series of different samples may be tested at the same time, some for one pathogen, some for another. It is particularly advantageous to test aliquots of a single sample simultaneously but separately for different pathogens, for example, to test each aliquot in a separate well of a microtitre plate for a different pathogen. This results in savings of time and money, and also reduces the risk of mistakes through loss or confusion of samples.

Accordingly, the present invention also provides a method for determining simultaneously but separately, antibodies specific to two or more different pathogens in liquid test samples, which comprises

(i) bringing each sample into contact with one of a plurality of units, for example, in a single module, each unit comprising a solid phase having immobilized antibodies to one or more classes of immuno-globulin, whereby immunoglobulins of the respective class or classes present in each sample are captured on the solid phase, the immobilized antibodies being of the same class or classes in all units of the module,

(ii) contacting each unit that has previously been contacted with sample with an antigen capable of binding selectively to an antibody specific for one of the pathogens under investigation, each antigen being provided with means capable of directly or indirectly providing a detectable signal, and

(iii) determining any resulting immunoglobulin-antigen complex on the solid phase.

The number of different units used and hence antigens used will depend on the number of pathogens under investigation. Each unit may be, for example, one of the wells of a microtitre plate, or a vessel containing beads.

Alternatively, each unit may be a defined region on a membrane, strip, sheet, film or coating, especially when presented in an assay device. Devices having predetermined regions for capture of components from a sample under investigation are known per se. At its simplest, an assay strip device for a simultaneous but separate assay according to the present invention may comprise, for example, a strip of bibulous material having a band of capture immunoglobulin across the width of the strip, called herein the antibody capture zone. A band is preferably divided into sections by areas not coated with capture immunoglobulin. Each section of immobilized immunoglobulins may be considered to be a unit antibody capture zone.

An assay device for a simultaneous but separate assay is, for example, as descibed above in relation to combination assays.

The versatility of an assay device of the present invention for a simultaneous but separate assay for different pathogens provides particular advantages for both manufacturer and user. A major advantage is that one device, provided with a number of defined antibody capture zones, can be supplied together with a plurality of antigen reagents for simultaneous testing for a number of different antibodies in a sample. As before, the manufacturer has the advantage that only one device need be made for all analytes. The user, too, has the advantage of requiring only one device, and has the further advantage of free choice of the combination of analytes to be determined.

As indicated above, a simultaneous but separate assay of the

present invention for different pathogens may be carried out for the investigation of any particular combination of pathogens in a sample. For example, a sample from a patient suffering from hepatitis may be tested simultaneously for hepatitis A, hepatitis B anti-core antibodies and HCV; a sample from a patient with non-specific urethritis may be tested for gonorrhoeae, chlamydia and candida.

In a variant of the simultaneous but separate assay of the present invention, instead of each unit having immobilized antibodies of the same class or classes, one or more of the units may have a different class of immunoglobulin immobilized thereon. For example, there may be provided a set of two units, one unit having immobilized anti-IgG and the other immobilized anti-IgM. A series of simultaneous assays for a particular antibody carried out over a period of time using such a two-unit set on samples obtained from the same individual is particularly useful for detecting early infection and for following the course of a disease, especially for following seroconversion, for example, of HIV or HCV. A set of three units, one having immobilized anti-IgG, one having immobilized anti-IgM and the third having immobilized anti-IgA is particularly useful for determining if a baby has HIV: a baby's blood has both the baby's own antibodies and maternal antibodies, so using conventional tests it is difficult to determine if the baby does have its own HIV anti-bodies, which are indicative of infection.

A further example of the use of more than one class of antibody is in the so-called "TORCH" test carried out in many countries on pregnant women. The pathogens are selected from rubella, toxoplasmosis, CMV and herpes. The combination of choice varies from country to country. The rubella test should include an IgM assay in order to detect a recent infection, which is potentially dangerous. Accordingly, for a "TORCH" assay involving rubella, at least one of the units in the set used should have an anti-IgM coating.

A module comprising two or more units having immobilized antibodies against different classes of immunoglobulins is itself part of the present invention. Accordingly, the present invention provides a module comprising two or more units for use in a simultaneous but separate assay for different pathogens, the units comprising immobilized antibodies against immunoglobulins, at least one unit having having immobilized antibodies directed against a different class of immunoglobulins from the immobilized immunoglobulins in the other units, for example, one unit may have immobilized anti-IgM and one or more units may have immobilized IgG. In another embodiment, a module comprises three units, one unit having immobilized anti-IgG, one unit having immobilized anti-IgM and the third unit having immobilized anti-IgA.

A module comprising two or more units may be present in an assay device, for example, as an arrangement of two or more antibody capture zones, for example, an IgG capture zone and an IgM capture zone. Such an assay device may be used for a simultaneous but separate assay for different pathogens, for example, for any of the particular uses described above.

Alternatively, a module may comprise two or more sets of units (each set comprising identical units). A series of sets of different units may be presented as a module. For example, a set of units may be presented as a strip of microwells. Such strips may be assembled as desired into modules comprising two or more sets of units, for example, a module may comprise two strips of microwells, one coated with anti-human IgG and one coated with anti-human IgM. A third strip, having immobilized anti-human IgA may be added. Strips of microwells i.e. sets of units, may be assembled as desired to form a conventional microtitre plate. For example, a plate may have alternate rows or columns of anti-IgM and anti-IgG coated microwells, for example, for following seroconversion. Again, there are manufacturing cost advantages and also the advantage of versatility of use, particularly for strips of microwells or analogous sets of units.

Unless otherwise specified, the following description applies to both the combination and the simultaneous but separate assays of the present invention:

For clinical testing, the liquid test sample may be any body fluid, for example, blood, plasma, serum, saliva, urine, cerebro-spinal fluid, milk, lymph fluid or tears. Alternatively, a solid sample, for example, of cells or tissue may be brought into liquid form for testing, for example, as tissue exudate.

For blood screening (which is advantageously carried out as a combination assay) the pathogens of interest are so-called blood viral pathogens (HIV, hepatitis B and hepatitis C, optionally also HTLV, CMV and EBV), and also syphilis. For clinical testing, a very much wider range of pathogens are of interest, including the above organisms and also other viruses, bacteria and other types of pathogenic organisms. By way of example, but non-limiting, are the following:

Rubella, measles, Herpes (simplex and genitalis), Chlamydia, Gonorrhoeae, hepatitis A, chickenpox, mumps, human parvovirus, Mycobacteria tuberculosis, Mycobacteria leprae, Mycobacteria avium, Staphylococcus aureus, Listeria monocytogenes, Bacillus anthracis (antigen/toxins), Actinomycetes (for example, Streptomyces, Nocardia, Rhodococcus), Salmonella typhi, Yersinia enterocolifica, Helicobacter pylori, Campylobacter jejuni, Pseudomonas mallei and pseudomallei, Pseudomonas aeruginosa, Legionella pneumophila and spp, Francisella tolarensis, Brucella melitenis, Mycoplasma pneumoniae, Leptospira interrogans, Borelia spp, Treponema pallidum, Candida albicans, and diseases caused by protozoal pathogens, for example, amoebiasis, babesiosis, Chagas' disease, leishmaniasis, malaria and toxoplasmosis.

The immobilized anti-immunoglobulins may be of one or more classes, that is to say, of the IgG, IgM and IgA classes. As mentioned above, IgG is the most abundant immunoglobulin in serum. IgM is the first immunoglobulin produced in response to

infection, so provides an earlier indication of infection than does IgG. For testing blood (plasma or serum), generally either IgG or IgM may be used alone, according to the purpose of the assay, or a mixture of anti-IgG and anti-IgM may be used in one unit for a combination assay or anti-IgG and anti-IgM may be presented in separate units for a simultaneous but separate assay. For assaying saliva, it is also preferable to capture IgG and/or IgM. For assaying urine, anti-IgG is preferably used, optionally in combination with IgM and/or IgA.

For those forms of assay of the present invention which utilise the two-component bead/tube format, it is possible to increase even further the adaptability of the assay of the present invention by immobilising one or two classes of anti-immunoglobulins on the inner surface of a plastics tube and immobilizing one or two different classes of anti-immunoglobulins on beads, the beads to be used in the tube. For example, anti-IgG may be coated on one of the two components, and anti-IgM on the other.

Furthermore, a combination bead/tube format may be used and, after contacting the tube and bead with the sample, the bead may be removed and contacted with one antigen and the tube contacted with another antigen, enabling two antibody assays to be carried out on a single sample.

Although both blood screening and clinical testing have been described above, there is no difference in the principle of the assays used, or in the type of materials used. For blood screening a particular range of analytes are specified by the specific national regulatory authorities, and the testing tends to be more fully automated than clinical testing simply because of the large numbers of identical tests. However, it is to be understood that, throughout the specification, unless specified otherwise, all descriptions relate to assays of the present invention in general, and include assays carried out on assay devices as well as those carried out on, for example, microtitre

plates or beads.

The assays of the present invention may be carried in a conventional manner see, for example, "ELISA and Other Solid Phase Immunoassays Theoretical and Practical Aspect", Eds. Kemeny D.M. & Challacombe S.J. Immunoglobulins may be immobilized on a solid phase, for example, by contacting the solid phase with a solution of the immunoglobulins at an appropriate concentration and pH, for example, at a pH within the range of from 7 to 11, especially from 9 to 10. A buffer is preferably used, for example, a sodium carbonate/sodium bicarbonate buffer. As indicated above, suitable immunoglobulin preparations are available commercially, and a suitable dilution of a commercial antibody solution is, for example, 1:200 to 1:4000 v/v.

A labelled antigen reagent (conjugate) may be produced by any of a variety of methods see for example, Kemeny & Challacombe, loc cit. Antigen-enzyme conjugates are generally used.

Samples may be diluted, for example, blood or plasma samples may be diluted 1 in 1 v/v, for example, 50$\mu$l of sample is added to 50$\mu$l of diluent in a microwell. It should be noted that any sample diluent used should not contain human immunoglobulins of the class or classes that are to be captured. In a microtitre plate or bead format assay, the sample is then generally incubated with the solid phase. The temperature and time of incubation are interdependent, a longer time being required at a lower temperature. Typical incubation conditions are one hour at 37°C. After incubation, the plate or beads should be washed thoroughly prior to incubation with the antigen reagent (conjugate). Typical incubation conditions for the conjugate stage are 30 minutes at 37°C. After incubation with the conjugate, there is a further washing step, followed by incubation with the substrate for the enzyme in the case of an ELISA. Again, 30 minutes at 37°C are typical incubation conditions. A stop solution is generally added at the end of the incubation. In the case of an ELISA, results are generally

obtained by reading the absorbence of each unit in a spectrophotometer. If another labelling system is used, the method is modified accordingly, for example, in the case of a radioimmunoassay or a fluorescence assay, results are obtained after incubation with a radiolabelled or fluorescent antigen conjugate. In the case of coloured particles, it may be possible to determine positive and negative results by eye.

In the case of an assay device for use outside a laboratory, coloured particles are particularly useful as label, since a positive or negative result can be determined by eye with a minimum number of reaction steps. For a more sensitive assay, an ELISA system may be used.

The present invention also provides a kit comprising
(a) a solid phase, especially plastics beads or a microtitre plate, carrying immobilized antibodies to one or more classes of immunoglobulin, especially a mixture of anti-IgG and anti-IgM antibodies,
(b) two or more antigen reagents each capable of binding selectively to an antibody specific for one of the pathogens under investigation, and each antigen being provided with means capable directly or indirectly of providing a detectable signal, and optionally, one or more of the following:
(c) positive and negative control reagents, washing solutions and diluents.

The kit may be provided as such, or the antibody-coated component and the antigen reagent component may be provided separately.

The present invention also provides a solid phase suitable for use in an immunoassay, on which is immobilized a mixture of anti-IgG and anti-IgM antibodies. The mixture of antibodies may additionally comprise anti-IgA antibodies. The antibodies are especially anti-human antibodies. The solid phase is, for example, any of the solid phases described above, for example, microtitre plates and beads, and includes those suitable for use

in assay devices.

The terms "detection" and "determination" are both used herein to denote qualitative, quantitative and semi-quantitative assays for pathogens.

The use of the immunoglobulin capture format for the determination of antibodies to a plurality of pathogens in different samples ("combination assay") or for the simultaneous but separate determination of a plurality of different antibodies in aliquots of the same sample ("simultaneous assay") gives advantages both in time and cost. The potential versatility of the format, in that multiple pathogens can be determined simultaneously either in one unit or in a series of parallel units simply by choice of antigen reagent, had not been appreciated previously. The very real practical and economic advantages of being able to use a universal antibody-coated means, for example, antibody-coated microtitre plates, antibody-coated beads, and immobilized antibodies in an assay device, for any antibody assay should not be underestimated.

It will, of course, be appreciated that the present invention is not limited to the detection of pathogens. The present invention may be applied to the detection of any antibody of interest, whatever the nature of the antigen that gives rise to the antibody, for example, non-pathogen-associated antibodies. Examples of conditions giving rise to such antibodies include autoimmune diseases and allergies, for example, non-organ-specific autoimmune diseases, for example, rheumatoid arthritis, lupus erythematosus and rheumatic fever, and organ-specific autoimmune diseases and diseases considered to have some autoimmune involvement, for example, autoimmune diseases of the thyroid, myasthenia gravis, autoimmune haemolytic anaemias, multiple disseminated sclerosis, aphthous ulcer, pernicious anaemia and ulcerative colitis. All that is required is an entity capable of binding specifically to any antibody of interest.

Accordingly, it is to be understood that the teachings of present specification are as relevant to the detection of non-pathogen related antibodies as they are to antibodies to pathogens, and that the present invention includes all such embodiments.

The present invention may be used in the detection of determination of human or animal pathogens, and finds both human and veterinary applications, including applications in the meat trade.

The following non-limiting Examples illustrate the invention.

## EXAMPLES

### Reagents

The following reagents were used for the assay described below:

1. Solid phase: 96-well microtitre plate (Nunc) coated with a mixture of polyclonal anti-human IgG (DAKO) and polyclonal anti-human IgM (DAKO). (Nunc products are available from Life Technologies, PO Box 35, Washington Road, Abbotts Inch Industrial Estate, Paisley, Renfrewshire, TA3 4EF, Scotland, and DAKO products from DAKO, 16 Manor Courtyard, Hughenden Avenue, High Wycombe, Bucks HP3 5RE, England).
2. HIV-1 recombinant protein comprising core and envelope antigens from the CBL-1 isolate of HIV-1 (Sattentau Q.J. et al. (1986) Science 234 1120) conjugated to HRP (horse-radish peroxidase) ("HIV-1 conjugate").
3. HIV-2 peptide comprising gp36 envelope antigen, conjugated to HRP ("HIV-2 conjugate").
4. Hepatitis core antigen conjugated to HRP
5. Positive samples:
   a) HIV-1, internal reference No. 4034, diluted in HIV-1 negative serum
   b) HIV-2, internal reference No. 91/174 diluted in HIV-2 negative serum
   c) anti-HBc core positive samples diluted in HBc negative

serum

6. Negative samples: normal human serum obtained from donated blood.

Method

100μl of diluted samples (10 μl sample and 90μl sample diluent) were added to wells of a microtitre plate and incubated under humid conditions at 37°C for 60 minutes. The wells were then washed thoroughly five times with a wash solution, each wash step involving removal of the contents of each well by aspiration, filling the well with wash solution ((glycine borate buffer containing Tween), and soaking for 30 seconds. After the final wash step the contents of the well are removed and the wells are inverted and tapped dry on a paper towel or tissue. 50μl of a working strength solution of the relevant conjugate(s) in HEPES buffer containing bovine serum albumin and detergents were added to the wells either singly or in combination as described below and the plate was incubated at 37°C for 30 minutes under humid conditions. After a further wash step as described above, 100μl of substrate solution containing TMB (3,3',5,5'-tetramethylbenzidine) and hydrogen peroxide was added to each well, the plate was incubated at 37°C under humid conditions for 30 minutes and the reaction was then stopped using 50μl of 2M sulphuric acid. The absorbance in the wells was recorded at 450nm with 690nm as the reference wavelength.

EXAMPLE 1

A series of positive samples each containing one species of antibody of interest (anti-HIV-1, anti-HIV-2 and anti-HBc) were diluted and tested according to the above protocol using the HIV-1, HIV-2 and HBc conjugates described above, either alone or in various combinations as shown in the Tables below. The HIV-1 sample was serially diluted 1/40; 1/80; 1/160 and 1/320. The HIV-2 sample was diluted 1/32; 1/64; 1/128 and 1/256. A number of different HBc-positive samples were diluted 1/2.

(i) HIV-1 positive samples at increasing dilutions were tested each with the following conjugates: HIV-1; HIV-1+HIV-2; HIV-1+HBc; HIV-1+HIV-2+ HBc. The results are presented in Table 1.

TABLE I

| Sample -> | HIV-1 | HIV-1 | HIV-1 | HIV-1 |
|---|---|---|---|---|
| increasing | >3 | 2.897 | >3 | >3 |
| dilutions | 2.015 | 1.905 | 2.011 | 1.847 |
| | 1.282 | 1.148 | 1.214 | 1.129 |
| | 0.694 | 0.663 | 0.695 | 0.672 |
| | 0.41 | 0.378 | 0.412 | 0.378 |
| | 0.232 | 0.230 | 0.282 | 0.235 |
| negative | 0.058 | 0.061 | 0.068 | 0.076 |
| negative | 0.060 | 0.064 | 0.075 | 0.075 |
| Conjugate | HIV-1 | HIV-1+ HIV-2 | HIV-1+ HIV-2 | HIV-1+ HIV-2+ HBc |

(ii) HIV-2-positive samples at increasing dilutions were each tested with the following conjugates: HIV-2; HIV-2+HIV-1; HIV-2+HBc; HIV-1+HIV-2+HBc. The results are presented in Table 2.

TABLE 2

| Sample | HIV-2 | HIV-2 | HIV-2 | HIV-2 |
|---|---|---|---|---|
| increasing | 0.099 | 1.045 | 1.009 | 1.002 |
| dilutions | 0.588 | 0.635 | 0.619 | 0.619 |
| | 0.386 | 0.397 | 0.386 | 0.383 |
| | 0.232 | 0.252 | 0.249 | 0.251 |
| | 0.158 | 0.167 | 0.171 | 0.183 |
| | 0.111 | 0.124 | 0.126 | 0.135 |
| negative | 0.053 | 0.060 | 0.061 | 0.072 |
| negative | 0.053 | 0.062 | 0.063 | 0.075 |
| Conjugate | HIV-2 | HIV-2+ HIV-1 | HIV-2+ HIV-1 | HIV-2+ HIV-1+ HBc |

(iii) Six different HBc-positive samples were each tested with the following conjugates: HBc; HBc+HIV-1; HBc+HIV-2; HBc+HIV-1+HIV-2. The results are presented in Table 3.

TABLE 3

| Sample | HBc | HBc | HBc | HBc |
|---|---|---|---|---|
| | 0.956 | 1.072 | 0.970 | 0.926 |
| | 0.980 | 1.073 | 0.986 | 0.913 |
| | 0.994 | 1.053 | 0.966 | 0.032 |
| | 1.072 | 1.189 | 1.058 | 1.030 |
| | 1.062 | 1.177 | 1.066 | 1.025 |
| | 1.058 | 1.156 | 1.062 | 1.005 |
| negative | 0.056 | 0.070 | 0.061 | 0.074 |
| negative | 0.060 | 0.069 | 0.063 | 0.073 |
| Conjugate | HBc | HBc+ HIV-1 | HBc+ HIV-2 | HBc+ HIV-1+ HIV-2 |

Discussion

The results set out in Tables 1 to 3 above show clearly that the various antigen conjugates can detect the respective antibodies as effectively in combination as when used alone. There is little interference when two or even three conjugates are used. There is only a small increase in the background levels observed for the negative samples when two or more conjugates are used.

EXAMPLE 2

A series of samples A to D containing HIV-1, HIV-2 and HBc antibodies were prepared, with dilutions of the original single samples as shown in Table 4.

TABLE 4

| | HIV-1 | HIV-2 | HBc |
|---|---|---|---|
| A | 1/40 | 1/32 | 1/2 |
| B | 1/80 | 1/64 | 1/2 |
| C | 1/160 | 1/128 | 1/2 |
| D | 1/320 | 1/256 | 1/2 |

Samples A to D were then each tested with the following antigen conjugates: HIV-1; HIV-2; HBc; HIV-1+HIV-2; HIV-1+HBc; HIV-2+HBc; HIV-1+HIV-2+HBc. The results are given in Table 5.

TABLE 5

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| A | 1.314 | 0.961 | 0.361 | 1.602 | 1.415 | 1.185 | 1.689 |
| B | 0.840 | 0.619 | 0.386 | 1.232 | 1.075 | 0.848 | 1.482 |
| C | 0.519 | 0.368 | 0.401 | 0.804 | 0.803 | 0.650 | 1.102 |
| D | 0.298 | 0.216 | 0.402 | 0.465 | 0.614 | 0.526 | 0.828 |
| - ve | 0.062 | 0.049 | 0.052 | 0.059 | 0.059 | 0.054 | 0.068 |
| - ve | 0.055 | 0.049 | 0.050 | 0.054 | 0.057 | 0.052 | 0.064 |
| - ve | 0.054 | 0.047 | 0.049 | 0.054 | 0.057 | 0.051 | 0.062 |
| - ve | 0.053 | 0.046 | 0.050 | 0.052 | 0.057 | 0.052 | 0.063 |
| conjg | HIV-1 | HIV-2 | HBc | HIV-1 HIV-2 | HIV-1 +HBc | HIV-2 +HBc | HIV-1 HIV-2 HBc |

Discussion

These results show clearly that each of the antibodies can be detected selectiveally in the presence of the other antibodies, that is to say, the presence of the non-relevant antibodies does not affect the detection of the relevant antibodies. Furthermore, the results obtained at lower dilutions illustrate qualitatively the presence of the three specific antibodies, and at greater dilutions the results obtained are substantially additive.

The results obtained show that specificity is not compromised when antibodies to two different pathogens are detected, and also show that the sensitivity of the combination assay is remarkably high.

CLAIMS:

1. A method for determining specific antibodies to two or more different pathogens in a liquid test sample, which comprises

(i) contacting the sample with a solid phase on which are immobilized antibodies to one or more classes of immunoglobulin, whereby immunoglobulins of the respective class or classes present in the sample are captured on the solid phase,

(ii) simultaneously or sequentially contacting the solid phase, on which immunoglobulins from the sample have been captured, with two or more different antigens, each antigen being capable of binding selectively to an antibody specific for one of the pathogens under investigation, each antigen being provided with means capable of providing, directly or indirectly, a detectable signal, and

(iii) determining any resulting immunoglobulin-antigen complex formed on the solid phase.

2. A method as claimed in claim 1, wherein the immobilized anibodies are anti-IgG, anti-IgM or anti-IgA antibodies, or are a mixture of two or more thereof.

3. A method as claimed in claim 1, wherein a mixture of anti-IgG and anti-IgM antibodies are immobilized on the solid phase.

4. A method as claimed in any one of claims 1 to 3, wherein the immobilized antibodies are affinity purified polyclonal antibodies or monoclonal antibodies.

5. A method as claimed in any one of claims 1 to 4, wherein each antigen is labelled directly with means capable of providing a detectable signal.

6. A method as claimed in any one of claims 1 to 4, wherein the

means capable of providing a detectable signal for an antigen comprises

(i) an antibody Ab1 that is capable of binding to that antigen, the antibody Ab1 being itself provided with means capable of providing a detectable signal or

(ii) comprises an antibody Ab1 that is capable of binding to that antigen, and a second antibody Ab2, which is capable of binding to antibody Ab1, which antibody Ab2 is provided with means capable of providing a detectable signal.

7. A method as claimed in any one of claims 1 to 6, wherein the sample under investigation is a sample of donated blood.

8. A method as claimed in any one of claims 1 to 7, wherein the two or more pathogens under investigation are selected from HIV, hepatitis B virus, hepatitis C virus, HTLV, cytomegalovirus, Epstein Barr virus and syphilis.

9. A method as claimed in any one of claims 1 to 7, wherein the two or more pathogens under investigation are selected from Rubella, measles, Herpes (simplex and genitalis), Chlamydia, Gonorrhoeae, hepatitis A virus, chickenpox, mumps, human parvovirus, Mycobacteria tuberculosis, Mycobacteria leprae, Mycobacteria avium, Staphylococcus aureus, Listeria monocytogenes, Bacillus anthracis (antigen/toxins), Actinomycetes Salmonella typhi, Yersinia enterocolifica, Helicobacter pylori, Campylobacter jejuni, Pseudomonas mallei and pseudomallei, Pseudomonas aeruginosa, Legionella pneumophila and spp, Francisella tolarensis, Brucella melitenis, Mycoplasma pneumoniae, Leptospira interrogans, Borelia spp, Treponema pallidum, Candida albicans, and diseases caused by protozoal pathogens.

10. A method as claimed in any one of claims 1 to 8, wherein the two or more pathogens under investigation are selected from rubella, toxoplasmosis, cytomegalovirus and herpes virus.

11. A method for determining simultaneously but separately, antibodies individually specific to two or more different pathogens in liquid test samples, which comprises

(i) bringing each sample into contact with one of a plurality of units in a single module, each unit comprising a solid phase having immobilized antibodies to one or more classes of immunoglobulin, whereby immunoglobulins of the respective class or classes present in each sample are captured on the solid phase, the immobilized antibodies being of the same class or classes in all units of the module,

(ii) contacting each unit of the module that has previously been contacted with sample with an antigen capable of binding selectively to an antibody specific for one of the pathogens under investigation, each antigen being provided with means capable of directly or indirectly providing a detectable signal, and

(iii) determining any resulting immunoglobulin-antigen complex om the solid phase.

12. A method as claimed in claim 11, wherein at least one unit comprises a solid phase having immobilized anti-IgG antibodies, at least one unit comprises a solid phase having immobilized anti-IgM antibodies and, optionally, at least one unit comprises a solid phase having immobilized anti-IgA antibodies.

13. A method as claimed in claim 11 or claim 12, wherein the samples under investigation are aliquots of a single sample.

14. A method as claimed in any one of claims 1 to 13, wherein the solid phase comprises beads, or the wells or cups of microtitre plates, or solid or hollow rods or pipettes, or particles; or comprises membranes, sheets, strips, films or coatings of a porous, fibrous or bibulous material, optionally incorporated in an assay device.

15. A module comprising two or more units for use in a simultaneous but separate assay for different pathogens, the units comprising immobilized antibodies against immunoglobulins, at least one unit having having immobilized antibodies directed against a different class of immunoglobulins from the immobilized immunoglobulins in the other units.

16. A module as claimed in claim 15, which comprises one unit having immobilized anti-IgM and one or more units having immobilized IgG.

17. A module as claimed in claim 15, which comprises three units, one unit having immobilized anti-IgG, one unit having immobilized anti-IgM and the third unit having immobilized anti-IgA.

18. A solid phase suitable for use in an immunoassay, on which is immobilized a mixture of anti-IgG and anti-IgM antibodies.

19. A solid phase as claimed in claim 18, on which anti-IgA antibodies are also immobilized.

20. A solid phase as claimed in claim 18 or claim 19, which comprises beads, or the wells or cups of microtitre plates, or solid or hollow rods or pipettes, or particles; or comprises membranes, sheets, strips, films or coatings of a porous, fibrous or bibulous material, optionally incorporated in an assay device.

21. A method for determining specific antibodies to two or more different antigens in a liquid test sample, which comprises

(i) contacting the sample with a solid phase on which are immobilized antibodies to one or more classes of immunoglobulin, whereby immunoglobulins of the respective class or classes present in the sample are captured on the solid phase,

(ii) simultaneously or sequentially contacting the solid phase,

on which immunoglobulins from the sample have been captured, with two or more different antigens, each antigen being capable of binding selectively to an antibody specific for one of the pathogens under investigation, each antigen being provided with means capable of providing, directly or indirectly, a detectable signal, and

(iii) determining any resulting immunoglobulin-antigen complex formed on the solid phase.

22. A method for determining, simultaneously but separately, antibodies specific to two or more different antigens in liquid test samples, which comprises

(i) bringing each sample into contact with one of a plurality of units in a single module, each unit comprising a solid phase having immobilized antibodies to one or more classes of immunoglobulin, whereby immunoglobulins of the respective class or classes present in each sample are captured on the solid phase, the immobilized antibodies being of the same class or classes in all units of the module,

(ii) contacting each unit of the module that has previously been contacted with sample with an antigen capable of binding selectively to an antibody specific for one of the pathogens under investigation, each antigen being provided with means capable of directly or indirectly providing a detectable signal, and

(iii) determining any resulting immunoglobulin-antigen complex om the solid phase.

23. A method as claimed in claim 21 or claim 22, wherein the antibodies under investigation are non-pathogen associated antibodies.

24. A method as claimed in claim 21 or claim 22, wherein the

antibodies under investigation are autoimmne antibodies or antibodies associated with allergies.

25. A method as claimed in any one of claims 21 to 24, having the parameters defined in any one of claims 2 to 6, 11 and 12.

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/00788

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 5 G01N33/543 G01N33/569 G01N33/576

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 4 August 1994 | 17.08.94 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Hitchen, C |

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